Microbial inositol polyphosphate metabolic pathway as drug development target.

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Abstract

Inositol polyphosphates are a diverse and multifaceted class of intracellular messengers omnipresent in eukaryotic cells. These water-soluble molecules regulate many aspects of fundamental cell physiology. Removing this metabolic pathway is deleterious: inositol phosphate kinase null mutations can result in lethality or substantial growth phenotypes. Inositol polyphosphate synthesis occurs through the actions of a set of kinases that phosphorylate phospholipase-generated IP_3 to higher phosphorylated forms, such as the fully phosphorylated IP_6 and the inositol pyrophosphates IP_7 and IP_8. Unicellular organisms have a reduced array of the kinases for synthesis of higher phosphorylated inositol polyphosphates, while human cells possess two metabolic routes to IP_6. The enzymes responsible for inositol polyphosphate synthesis have been identified in all eukaryote genomes, although their amino acid sequence homology is often barely detectable by common search algorithms. Homology between human and microbial inositol phosphate kinases is restricted to a few catalytically important residues. Recent studies of the inositol phosphate metabolic pathways in pathogenic fungi (*Cryptococcus neoformans*) and protozoa (*Trypanosome brucei*) have revealed the importance of the highly phosphorylated inositol polyphosphates to the fitness and thus virulence of these pathogens. Given this, identification of inositol kinase inhibitors specifically targeting the kinases of pathogenic microorganisms is desirable and achievable.

Keywords:

Kinase; IPMK; metabolism; inhibitors; pathogen; ITPK1.

Introduction

Cells, whether part of a multicellular organism or independent beings, must adapt to external inputs to survive and reproduce. A unicellular organism must respond to the ever-changing external environment. A metazoan or plant cell, often highly specialised, must perform unique tasks in harmony with the other cell types; thus its physiology is controlled not only by external factors, such as nutritional input, but also from signals (for example mechanical, hormonal) deriving from other cells of the organism. Signal transduction mechanisms are used to integrate the received inputs and to coordinate the appropriate physiological answers. Misfiring, causing improper physiological responses, often affects growth rate, which can lead to cancerous development, or in the case of unicellular organisms can cause decreased fitness and ultimately death. Cell biology textbooks show us numerous signal transduction pathways: protein phosphorylation cascades, nuclear receptors, cAMP signalling etc. Inositol phosphates, in their lipid-bound or cytosolic forms, represent perhaps one of the most complex systems of signalling molecules present in eukaryotic cells. The signal transduction pathways regulated by phosphorylated forms of inositol are more than the AKT phosphorylation cascade activated by synthesis of the lipid PIP_3 (Hawkins et al., 2006) or the phospholipase-C (PLC) generation of the calcium release factor $I(1,4,5)P_3$ (Yang et al., 2013). Although less well-known than their lipid counterparts, the soluble 'cytosolic' inositol polyphosphates constitute a large metabolic network of signalling molecules with unexpected features (Irvine and Schell, 2001). Starting from the calcium release factor IP_3, specific kinases generate an array of phosphorylated molecules leading to the synthesis of IP_6 (inositol hexakisphosphate, also known as phytic acid), with a fully phosphorylated inositol ring. More phosphorylated forms exist: called inositol pyrophosphates, they possess seven (IP_7, diphosphoinositol pentakisphosphate), eight (IP_8, bis-diphosphoinositol tetrakisphosphate) and likely even more phosphate groups attached to the six-carbon inositol ring (Losito et al., 2009; Pisani et al., 2014). Depleting the cell of inositol pyrophosphates, by destroying the metabolic pathways that lead to their synthesis, has vast phenotypic consequences, since these molecules control numerous cell biological processes. This pleiotropy of functions suggests that inositol pyrophosphates control some fundamental aspect of cell physiology. Indeed, these molecules are emerging as important players in regulating basic energetic metabolism (Szijgyarto et al., 2011) through their ability to control phosphate homeostasis (Wild et al., 2016) (for review see (Azevedo and Saiardi, 2017; Shears, 2017; Wilson et al., 2013)).

While the AKT signalling cascade, generated from the lipid PIP_3, is largely characteristic of metazoa, the inositol phosphate metabolic network that leads to the synthesis of the inositol pyrophosphates is present with some variance in virtually all eukaryotic cells (Livermore et al., 2016).

The simplicity of and easy genetics and biochemistry of the budding yeast *Saccharomyces cerevisiae* have been instrumental in identifying the different inositol phosphate kinases that lead to the synthesis of IP_7 and IP_8 from PLC-generated $I(1,4,5)P_3$. In budding yeast, the sequential action of Arg82 (Ipk2 or inositol phosphate multikinase IPMK), IPK1 (IP5-2K), Kcs1 (IP6K) and Vip1 (PPIP5K) leads to the conversion of $I(1,4,5)P_3$ to IP_8. Only two of the known inositol polyphosphate kinases, IP_3-3K and ITPK1, have not been identified using the budding yeast, because they are actually absent from its genome. Undiscovered kinases may also still exist: in the amoeba *Dictyostelium discoideum* the synthesis of highly phosphorylated inositol phosphates occurs directly from inositol and not from PLC-generated IP_3 (Stephens and Irvine, 1990). The metazoa specific IP_3-3K is linked to calcium signalling, since it specifically converts $I(1,4,5)P_3$ to IP_4, switching off calcium release (Schell, 2010). On the other hand ITPK1 converts a different isomer of IP_3, namely $I(1,3,4)P_3$, to IP_5. Together these two kinases create a metabolic pathway leading to highly phosphorylated inositol such as IP_5, IP_6, and inositol pyrophosphates that is absent from yeast (see below) and many protozoa.

The identification of the inositol phosphate metabolic pathway in pathogenic fungi such as *Cryptococcus neoformans* (Lev et al., 2013; Lev et al., 2015; Li, C. et al., 2016) and pathogenic protozoa such *Trypanosoma brucei* (Cestari et al., 2016; Cordeiro et al., 2017) revealed the importance of these signalling molecules to the fitness of these organisms. The generation of inositol phosphate kinase null mutants results in multiple defects, including lethality or substantial growth delay phenotypes. Interestingly, these pathogens possess an array of inositol phosphate kinases similar to *S. cerevisiae*, and lack the alternative metazoan-specific synthetic pathway. Furthermore, the homology between the inositol phosphate kinases in these pathogens and their human counterparts is restricted to a few key amino acids, with an identity index below 30%. These three features: 1) the importance of highly phosphorylated inositol phosphates for the fitness of pathogenic organisms; 2) the presence, in human cells, of an alternative metabolic pathway leading to IP_6 and 3) the low amino acid homology existing between the human and the pathogenic kinases, points towards the microbial inositol polyphosphate metabolic pathway as a drug development target. Developing new treatments to fight eukaryote pathogens such as fungi or trypanosomes is

particularly challenging since the mammalian host is also eukaryotic and therefore with a similar cellular physiology. Targeting unique feature of each pathogen's physiology, such as the cell walls of pathogenic fungi, has led to the development of drugs that are often limited by species specificity. Drug resistance is also emerging (Prasad et al., 2016) and it has become urgent and imperative to identify novel targetable microbial pathways. Therefore, screening to identify inositol phosphate kinase inhibitors targeting specifically the kinases of pathogenic microorganisms, is not only achievable but also extremely desirable.

The inositol polyphosphate metabolic pathways synthesizing IP_6

For comprehensive reviews on the inositol polyphosphate metabolic network, we suggest the following essays (Irvine and Schell, 2001; Shears et al., 2012; Tsui and York, 2010). Here we briefly summarise the parts of the inositol phosphate metabolic network important to our argument. Historically, inositol phosphates acquired substantial research interest after the discovery that the hydrolysis of the lipid $PI(4,5)P_2$ by PLC generates two second messengers: diacylglycerol (DAG) and the calcium release factor $I(1,4,5)P_3$ (Figure 1). While the majority of $I(1,4,5)P_3$ is dephosphorylated and thus recycled back to inositol, inositol triphosphate also represents the building block on which the synthesis of higher phosphorylated inositol polyphosphates takes place. Two routes from $I(1,4,5)P_3$ eventually generate the inositol pentakisphosphate $I(1,3,4,5,6)P_5$. The first route, which we call the ITPK1 path, is present in metazoans and therefore humans, where IP_3-induced calcium signalling plays a predominant role in cell regulation. $I(1,4,5)P_3$ is rapidly metabolised by IP_3-3K that generates the inositol tetrakisphosphate $I(1,3,4,5)P_4$ by phosphorylating position three of the inositol ring (metabolic pathways depicted in Figure 2). This IP_4 is dephosphorylated by the inositol polyphosphate 5-phosphatase (5-PTase), generating the IP_3 isomer $I(1,3,4)P_3$. This is the primary substrate of ITPK1, mainly identified as a 5/6 kinase, which converts this IP_3 isomer to the 2-hydroxy $I(1,3,4,5,6)P_5$, usually the most abundant isomer of IP_5 in eukaryotic cells. ITPK1 also has an intrinsic 1-phosphatase activity that allows it to generate $I(3,4,5,6)P_4$, an inhibitor of calcium-activated chloride channels (Mitchell et al., 2008; Saiardi and Cockcroft, 2008; Shears, 2009).

The second route, which we call the IPMK path, is common to virtually all eukaryote organisms. The PLC-generated $I(1,4,5)P_3$ is phosphorylated at the 3 and 6 positions by IPMK, and is thus converted to the same IP_5 isomer that is generated by the ITPK1 path (Figure 2) (Odom et al., 2000; Saiardi et al., 1999). Human IPMK is unusual amongst the inositol phosphate kinases since it

can also phosphorylate the lipid PI(4,5)P_2 and generate PIP_3 (Resnick et al., 2005; Wang and Shears, 2017). Thus both metabolic routes starting from I(1,4,5)P3 converge at I(1,3,4,5,6)P_5. This is the substrate of IP5-2K (Ives et al., 2000) which generates the fully phosphorylated and most abundant inositol polyphosphate present in cells, IP_6. In metazoan cells, where both IPMK and ITPK1 paths coexist, their relative importance for the synthesis of IP_5 and IP_6 may depend on the relative expression of the IPMK and ITPK1 kinases. Downregulation by RNAi of ITPK1 in HeLa cells led to a substantial decrease in IP_5 and IP_6 levels (Verbsky et al., 2002). Conversely, stem cells generated from ipmk$^{-/-}$ knockout mice blastocysts show a 90% reduction in IP_6 levels (Frederick et al., 2005). Similar analysis, performed by the newly developed PAGE analysis (Wilson et al., 2015), on ipmk$^{-/-}$ mouse embryonic fibroblasts (MEF), reveals a reduction in IP_6 level of ~80% (Figure 3). In plants, where both IPMK (Ipk2) and ITPK1 kinases are present, an *Arabidopsis thaliana* ipmk deficient plant line (*atipk2*) produced seedlings with reduced but not ablated IP_6 levels (Stevenson-Paulik et al., 2005). Therefore, in mammals or plants, where both IPMK and ITPK1 are present, the elimination of either one does not lead to complete IP_6 removal. This is not the case in yeast where only the IPMK path is present: in the *S. cerevisiae arg82*Δ mutant, the non-metabolised IP_3 accumulates, and higher phosphorylated forms of inositol are absent (Hatch et al., 2017; Saiardi et al., 2002).

IPMK and ITPK1, two different inositol multikinases

Based on sequence homology and on crystal structure, the inositol phosphate kinases can be categorized into four different families: the inositol 5/6 kinase or ITPK1 family; the IP5-2Ks (*S. cerevisiae* Ipk1); the PPIP5K (*S. cerevisiae* Vip1) proteins that have a dual domain structure with both a kinase and a phosphatase domain (Pohlmann et al., 2014; Shears et al., 2017); and the Inositol Phosphate Kinase "IPK" superfamily (Pfam 03770) which includes the subgroups IP_3-3K, IPMK (S. cerevisiae Arg82 or Ipk2) and the IP6Ks (S. cerevisiae Kcs1). Members of this superfamily share signature motifs and assume the same overall three-dimensional fold. Six crystal structures of IPK family members have been resolved so far (Endo-Streeter et al., 2012; Gonzalez et al., 2004; Holmes and Jogl, 2006; Miller and Hurley, 2004; Wang et al., 2014; Wang and Shears, 2017), including IPMK from *S. cerevisiae* (Holmes and Jogl, 2006), *Arabidopsis thaliana* (Endo-Streeter et al., 2012) and *Homo sapiens* (Wang and Shears, 2017). Predictably, the core structural element of the three resolved IPMK structures are virtually identical to each other. The IPMK fold is defined by three regions: an N-terminal domain, a larger C-terminal domain, and a small inositol-binding domain consisting of two alpha-helices. While the overall domain fold is conserved between the

five resolved structures of IPK family members, the size and organization of the inositol-binding domain defines the substrate specificity. The small inositol-binding domain of IPMK offers fewer constraints to substrate binding, explaining the promiscuity of this enzyme. Nevertheless, the IPMK structures highlighted that, outside of the key elements, important differences exist between IPMKs from different species. These dissimilarities are largely localized in the external loops and in species-specific inserts. Analysis of IPMK primary amino acid sequences reveals an identity between the human enzyme and its microbial counterparts below 30%. Multiple sequence alignments of human with pathogenic microbial organism IPMKs demonstrates the conservation of key structural amino acids in an overall low conservation of primary sequence homology (Figure 4).

Several inositol polyphosphate kinases display substrate promiscuity, a conservative but metabolically proliferative measure on behalf of evolution whose likely function is to allow rapid conversion of inositol polyphosphates into other inositol entities. Though IPMK is considered the typical multipurpose kinase (Kim et al., 2016; Resnick and Saiardi, 2008), ITPK1 is even more catalytically flexible given its 1-phosphatase/1-kinase activities. The crystal structure of *Entamoeba histolytica* ITPK1 revealed a fold and specificity determinants entirely dissimilar from IPMK (Miller et al., 2005). The crystal obtained with its substrates revealed that the ATP γ-phosphate is positioned equidistantly from the 5- and 6-hydroxyl groups of the enzyme's main substrate $I(1,3,4)P_3$ explaining the dual site specificity of this kinase. Furthermore, more than the stereochemistry of the inositol ring, the phosphate groups determine substrate specificity; up to 18 different substrates could engage the inositol-binding pocket (Miller et al., 2005), further explaining the catalytic flexibility of this kinase. The different tertiary folds of IPMK and ITPK1 emphasize that these two enzymes are not related to each other. Thus, evolution has developed two different inositol "multikinases", both important to ultimately synthesize the same isomer of IP_5, (Figure 2) precursor of IP_6 and of the inositol pyrophosphates. This existence, in metazoan and many eukaryotes, of two metabolic routes for the synthesis of the same highly phosphorylated forms of inositol, underscores the importance of these molecules to eukaryotic cell physiology (Banfic et al., 2016; Szijgyarto et al., 2011; Wild et al., 2016).

The inositol polyphosphate pathway of pathogenic microorganisms

In only two pathogenic microorganisms have the metabolic pathways that lead to the synthesis of inositol pyrophosphates been characterised in detail. These are the fungus *C. neoformans*, responsible for invasive fungal infections in immune depressed patients, and the

parasite *T. brucei*, which causes African trypanosomiasis or sleeping sickness and is the experimental model for other trypanosome diseases, such as Chagas disease caused by *T. curzi*. Here we will summarise these studies.

C. neoformans is an opportunistic yeast pathogen causing serious disease and mortality in immunocompromised individuals. It is the principal cause of meningitis in HIV immune depressed patients, causing half a million deaths annually (Denning, 2016). Thanks to the efforts of the group of Dr J.T. Djordjevic *C. neoformans* is the only fungal pathogen with extensive characterisation of the inositol polyphosphate metabolic pathway (Lev et al., 2013; Lev et al., 2015; Li, C. et al., 2016). This yeast possesses six inositol phosphate kinases, one more than *S. cerevisiae*. Two are homologous to IPMK (Arg82 or Ipk2), called Arg1 and Arg2. However, only Arg1 is functionally similar to Arg82 since the null mutant *arg1*Δ accumulates IP_3 as in the budding yeast equivalent. The *arg2*Δ mutant does not accumulate IP_3 and is phenotypically identical to wild type (WT) yeast (Lev et al., 2013). *C. neoformans* Ipk1 converts IP_5 to IP_6, and the *ipk1*Δ strain, as in the budding yeast counterpart, accumulates IP_5 that is converted to PP-IP_4 (diphosphoinositol tetrakisphosphate) by the action of Kcs1. The presence of PP-IP_4 supports the fitness of *ipk1*Δ likely by fulfilling some of the functions of IP_7 (Li, C. et al., 2016). The IP_6 kinase, called as in budding yeast Kcs1, primarily generates IP_7 from IP_6. Finally, the PP-IP_5 kinase called Asp1 (*S. cerevisiae* Vip1) is responsible for IP_8 synthesis since the *asp1*Δ strain accumulates IP_7 and is IP_8 deficient (Lev et al., 2015). Phenotypically the IPMK mutant *arg1*Δ shows the greatest defects, followed by *kcs1*Δ. For many features *ipk1*Δ is similar to WT, as is the *asp1Δ* strain. Since the growth of *arg1*Δ strain was compromised at 37°C, the virulence of this strain was tested using *Galleria mellonella* larvae incubated at 30°C; these studies revealed that the virulence of *arg1*Δ is substantially attenuated (Lev et al., 2013). As *kcs1*Δ was able to grow at 37°C, the virulence of this strain was tested more directly in mice: 100% of infected mice survived the infection after a 50 day incubation period, while mice infected with the WT strain all perished (Lev et al., 2015). Conversely, the *asp1*Δ strain was as virulent as WT indicating that IP_8 plays only a minor role, if any, in *C. neoformans* virulence. Despite *arg1*Δ and *kcs1*Δ strains sharing many common phenotypes, involving cell wall integrity, urease synthesis, and melanin and mating filaments morphology, they are not phenocopies of each other. The *arg1*Δ mutant showed the greatest growth defect, besides being unable to grow at 37°C, and showed small capsules and thickened cell wall (Lev et al., 2013). Conversely, *kcs1*Δ has a mucoid appearance and large capsules. Although avirulent, *kcs1*Δ did establish a residual

asymptomatic lung infection that failed to disseminate to the central nervous system. The sugar composition of *kcs1*Δ capsules might explain this phenotype. The *kcs1*Δ mutant appeared to have reduced levels of mannoprotein, which is essential to induce monocyte and macrophage recruitment to the site of infection and for the uptake of the yeast by these cells (Lev et al., 2015). This is a crucial step for *C. neoformans* transportation through the blood stream and through the blood-brain barrier, where lethal meningitis develops.

In *T. brucei* three inositol phosphate kinases, one less than in *S. cerevisiae*, have been identified. This trypanosome possesses an IPMK homologous enzyme (TbIPMK) that converts IP_3 to IP_5, and an IP5-2K (TbIP5K) responsible for synthesizing IP_6 from IP_5. In this organism, only one enzyme, an IP6K (TbIP6K), synthesizes inositol pyrophosphates since the PPIP5K (Vip1) gene is absent in trypanosome genomes, as is any ITPK1 homologous enzyme (Cordeiro et al., 2017). The trypanosome proteins complement *S. cerevisiae* strains deficient in their corresponding orthologues, providing *in vivo* evidence that they encode functional enzymes involved in the synthesis of inositol polyphosphates (Cordeiro et al., 2017). A conditional knockdown has been generated for TbIPMK (Cestari et al., 2016). The analysis of these knockdown cells revealed that this kinase is essential for the bloodstream forms of the parasites (Cestari and Stuart, 2015), indicating that inositol polyphosphates are essential for this life stage. Furthermore, TbIPMK knockdown cells showed reduced infectivity (Cestari et al., 2016). TbIPMK mutants possess an altered basic metabolism since the synthesis of the polymeric form of phosphate, inorganic polyphosphate or polyP, is affected in these cells (Cordeiro et al., 2017). The TbIPMK mutant fails to accumulate this polymer into the main polyP storage compartment, the acidocalcisome. Thus, in both trypanosome and yeast the destruction of the inositol polyphosphate metabolic pathway affects polyP synthesis. Altered inositol polyphosphate metabolism possibly affects the trypanosome polyP-synthesizing enzyme TbVTC4 (Lander et al., 2013). Interestingly, the activity of the homologous yeast protein Vtc4 is regulated by inositol pyrophosphates interacting with its SPX protein domain (Azevedo and Saiardi, 2017; Wild et al., 2016). The knockdown strains for TbIP5-2K and TbIP6K have not yet been characterised.

These studies reveal that in both pathogenic yeast and trypanosomes alteration of inositol metabolism results in reduced fitness, and reduced or absent infectivity. Therefore, the inositol polyphosphate pathway of pathogenic microorganisms is a promising drug development target.

IPMK as the ideal targetable enzyme

The discussed studies further emphasize the importance of highly phosphorylated inositol phosphates to eukaryote cell physiology. In *T. brucei* the TbIPMK null mutant is lethal for the bloodstream form of the parasite, suggesting that inositol phosphate signalling is essential to control the parasite physiology inside the mammalian host. Several studies carried out in the infectious fungus *C. neoformans* have indicated that the inositol pyrophosphate IP_7, specifically the isomer 5PP-IP_5, is responsible for the pathogenicity of this yeast (Li, C. et al., 2016). The *kcs1Δ* null strain is avirulent. However, the IPMK mutant, *arg1Δ*, shows the stronger phenotype: this strain does not grow at 37°C and so cannot survive in the human host (Lev et al., 2013).

The yeast *S. cerevisiae* possesses only the IPMK path (Figure 2) leading to the synthesis of IP_6 and the inositol pyrophosphates. This is a common characteristic of all fungi. This eukaryote kingdom appears to have lost the ITPK1 gene. Therefore, not only *C. neoformans* but also other pathogenic fungi such as *Aspergillus fumigatus* and *Candida albicans* rely exclusively on the IPMK path for the synthesis of higher phosphorylated inositol species. Interestingly, ITPK1 is absent not only in the fungi kingdom but also from the genomes of many protozoa, which also do not possess the IP3-3K. Thus many other unicellular eukaryotes also rely exclusively on the IPMK path to synthesize IP_6, including, besides the trypanosomes, the pathogenic *Plasmodium falciparum* and *Giardia lamblia* (Saiardi lab unpublished observation).

Because of the demonstrated importance of IP_6 and the inositol pyrophosphates in eukaryote cell physiology, blocking their synthesis by pharmacologically targeting the IPMK path, in pathogenic organisms without ITPK1, will substantially affect the fitness of, if not eradicate, the pathogen. The relatively low homology existing between fungal or protozoan IPMK against the mammalian enzyme provides the possibility of developing selective IPMK inhibitors targeting only the pathogenic enzyme. Better still, the presence in human cells of the alternative ITPK1 path for IP_6 synthesis allows development of imperfect IPMK inhibitors. A drug that blocks the pathogenic IPMK but also partially affects the mammalian enzyme would likely be well tolerated, since the ITPK1 path will still synthesize IP_6 and compensate for a partial blockage of the IPMK path.

Drugs that inhibit the IPK family of enzymes have been described in the literature. The most famous is the purine analogue N2-(m-(trifluoromethyl)benzyl) N6-(p-nitrobenzyl)purine (TNP), originally developed as a IP3-3K inhibitor but subsequently identified as a IP6K inhibitor (Padmanabhan et al., 2009). However, TNP does not inhibit mammalian mouse IPMK (Kolozsvari et al., 2014). Mammalian IP3-3K enzymes are inhibited by several plant-derived or synthetic polyphenol compounds, while mammalian IPMK appears to be specifically inhibited by chlorogenic

acid (Mayr et al., 2005), a plant metabolite important for lignin biosynthesis that is commonly present in coffee. A relatively small (520 compounds) chemogenetic *in vivo* screening identified some *T. brucei* IPMK inhibitors (Cestari et al., 2016). However, their IC_{50} against IPMK enzymatic activity was higher (3.4-5.33 µM) than the EC_{50} for their growth inhibition (0.51-0.83 µM), suggesting that other targets might be involved in the sensitivity of *T. brucei* to those compounds. The effects of these inhibitors on human IPMK was not tested.

A systematic small molecule high-throughput inhibitor screen to identify specific pathogenic IPMK inhibitors has not been performed, although this is an area of intense interest (Li, Cecilia et al., 2016). To successfully perform such a screen for inositol kinase inhibitors, the assay to detect the enzymatic activity must be reliable and robust. Since inositol phosphates do not absorb light or emit fluorescence, the inositol phosphate kinase activity must be assayed by coupling it with secondary reactions, such as by coupling ADP production to NADH consumption via pyruvate kinase and lactate dehydrogenase reactions (Mayr et al., 2005). Even monitoring of the IPMK reverse reaction, i.e. the production of ATP measured using a luciferase assay, could be used. The recent identification that IP_5 and IP_6 induce a specific DAPI fluorescence (excitation at 420nm, emission at 550nm) while IP_3 does not (Kolozsvari et al., 2014) offers a straightforward alternative. It becomes possible to directly monitor IPMK-driven IP_5 synthesis by adding DAPI (4',6-diamidino-2-phenylindole) once the reaction is terminated. Monitoring this IP_5-induced DAPI fluorescence opens up the possibility of large scale screening for small molecule inhibitors of this class of enzymes.

The overwhelming importance of developing novel drugs to block fungal infection, to stop trypanosomiasis, or to fight malaria is obvious. It is not the objective of the authors to further emphasise what has been discussed hundreds of times before. The current essay highlights the importance of the inositol phosphates for the fitness and infectivity of pathogenic organisms, and points out the difference between human and pathogen inositol polyphosphate metabolism. Our arguments indicate that pathogenic microorganism IPMK is a possible, desirable and achievable drug target.

Acknowledgements

We thank Antonella Riccio for suggestions and helpful comments. We would also like to thank the core staff at the LMCB for facilitating our research.

Funding

This work was supported by the Medical Research Council (MRC) support to the MRC/UCL Laboratory for Molecular Cell Biology University Unit (MC_UU_1201814).

Abbreviations

PLC phospholipase-C; IP_3 inositol trisphosphate; IP_5 inositol pentakisphosphate; IP_6 inositol hexakisphosphate; IP_7 diphosphoinositol pentakisphosphate; IP_8 bis-diphosphoinositol tetrakisphosphate; DAG diacylglycerol; IPMK Inositol Phosphate Multi Kinase; ITPK1 inositol trisphosphate I(1,3,4)P_3 5/6 kinase or family; IP5-2Ks IP_5 two Kinase; PPIP5K; IPK Inositol Phosphate Kinase; IP_3-3K inositol trisphosphate I(1,4,5)P_3 3-kinase; 5-PTase Inositol polyphosphate 5-phosphatase; TNP N2-(m-(trifluoromethyl)benzyl) N6-(p-nitrobenzyl)purine; DAPI DAPI 4',6-diamidino-2-phenylindole.

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Figure legends

Figure 1: Schematic representation of the inositol cycle.

Many organisms or human cell lines can synthesize inositol monophosphate (IP) from glucose-6-phosphate (glucose-P), thanks to the activity of inositol phosphate synthase IPS or Ino1. The lithium-sensitive inositol monophosphatase IMPA, dephosphorylates IP, generating inositol. However, inositol can also be acquired from the extracellular space as a nutrient. Inositol is incorporated into lipids by the action of the phosphatidylinositol synthase (PI-synthase). The conversion of phosphatidylinositol (PI), first to PI(4)P (PIP), then to PI(4,5)P_2 (PIP_2) generates the phospholipase C substrate. PIP_2 hydrolysis by PLC generates two second messengers: the membrane-resident diacylglycerol (DAG) and the calcium release factor I(1,4,5)P_3 (IP_3). The latter is mainly dephosphorylated back to inositol, closing the "inositol cycle" (Berridge et al., 1989). However, IP_3 is also directly and indirectly converted to highly phosphorylated forms of inositol, thanks to the actions of an array of specific kinases and phosphatases (Irvine and Schell, 2001) (details in Figure 2).

Figure 2: Metabolic pathways synthesizing IP_6

The synthesis of higher phosphorylated inositol polyphosphates begins with the synthesis of Ins(1,4,5)P_3 by phospholipase C hydrolysis of the lipid PI(4,5)P_2. IPMK phosphorylates positions three and six of the inositol ring, converting I(1,4,5)P_3 to the 2-hydroxyl I(1,3,4,5,6)P_5 and defining the 'IPMK path' (blue arrows). However, especially in metazoa, where the IP_3-3K enzymes are present, position three of I(1,4,5)P_3 is rapidly phosphorylated to generate I(1,3,4,5)P_4, a substrate of the 5-phosphatase (5-PTase). Dephosphorylating position five generates a different IP_3 isomer, I(1,3,4)P_3, a substrate of ITPK1 that in a two step reaction generates Ins(1,3,4,5,6)P_5, defining the 'ITPK1 path' (red arrows). IP_6 is synthesized by the action of IP5-2K and itself becomes a substrate of the IP6K and PPIP5K enzymes, generating the inositol pyrophosphates. The *myo*-inositol ring is represented using the Mills projection. Each carbon is numbered in the PI(4,5)P_2 lipid while only the phosphorylated carbons are numbered in the two IP_3 structures, I(1,4,5)P_3 and I(1,3,4)P_3.

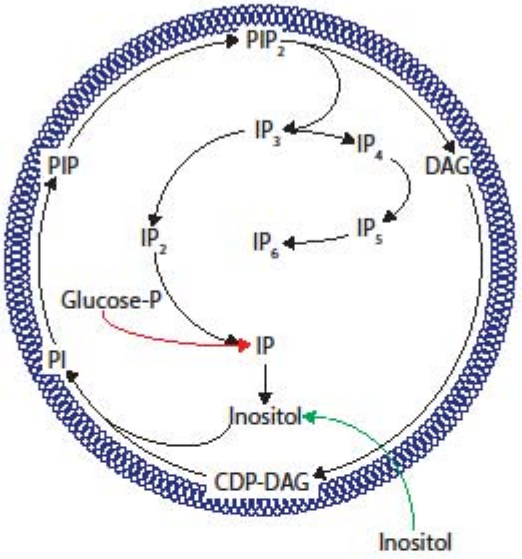
Figure 3: Levels of IP_6 in IPMK null MEF

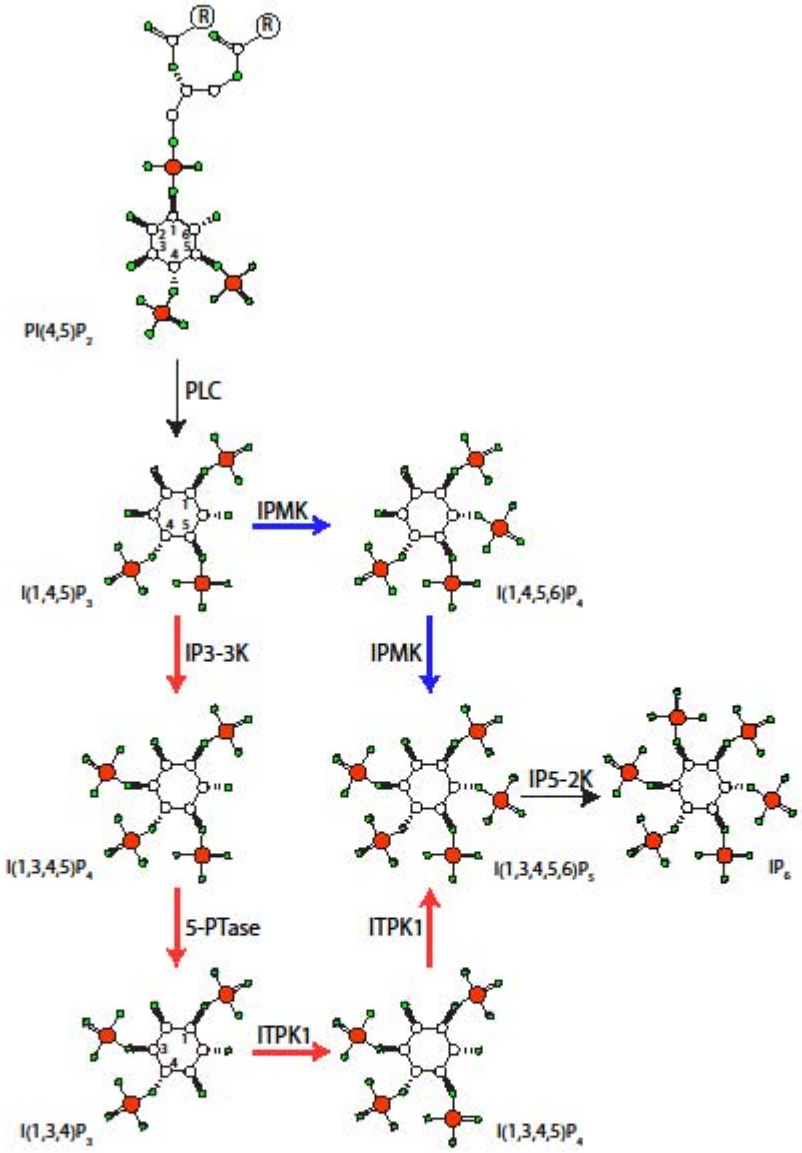
Inositol phosphates from two 14 cm dishes of 80 % confluent Mouse Embryonic Fibroblasts (MEF) (Maag et al., 2011) derived from wild type/parental (WT) or IPMK knockout mice ($ipmk^{-/-}$)

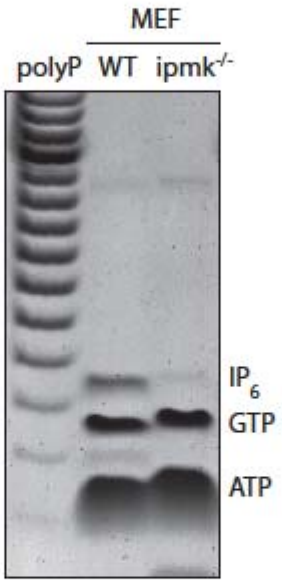
were extracted with perchloric acid and purified using TiO_2 beads before resolution by PAGE (Wilson et al., 2015). This analysis shows that ipmk$^{-/-}$ MEF have a reduced amount of IP_6. Densitometry analysis revealed an 80% reduction in IP_6 levels between WT and ipmk$^{-/-}$ MEF. The gel presented is representative of experiments performed three times. Inorganic polyphosphate, polyP, was used as position standard.

Figure 4: IPMK multi-alignment

Multi sequence alignment obtained using the cobalt software (https://www.ncbi.nlm.nih.gov/tools/cobalt/) of IPMK from human (*H. sapiens*), the yeast experimental model *S. cerevisiae* (Arg82 or Ipk2), the pathogenic yeast *C. neoformans* (Arg1) and the parasite *T. brucei* (TbIPMK). Asterisks denote conserved amino acid positions. Red boxes highlight the conserved signature motifs: the inositol-binding domain defined by PxxxDxKxG, the SSLL, and IDF regions. While these domains were originally identified through homology search (Saiardi et al., 1999) the subsequent crystal structures of IPK proteins have shown these signatures to be involved in substrate or cofactor binding (Gonzalez et al., 2004; Holmes and Jogl, 2006). The accession numbers of the sequences used were as follows: Human IPMK, NP_689416; *S. cerevisiae* Arg82, NP_010458; *C. neoformans* Arg1, XP_771784; *T. brucei* TbIPMK, XP_827490.


PIP2
IP3
IP4
DAG
PIP
IP2
IP6
IP5
Glucose-P
IP
PI
Inositol
CDP-DAG
Inositol


R
R
PI(4,5)P2
PLC
IPMK
I(1,4,5)P3
I(1,4,5,6)P4
IP3-3K
IPMK
IP5-2K
I(1,3,4,5)P4
I(1,3,4,5,6)P5
IP6
5-PTase
ITPK1
ITPK1
I(1,3,4)P3
I(1,3,4,5)P4


MEF
polyP
WT
ipmk-/-
IP6
GTP
ATP


H. sapiens
S. cerevisiae
C. neoformans
T. brucei